Gut, 1987, **28**, 70–74

In vitro effect of duodenal juice on R binders cobalamin complexes in subjects with pancreatic insufficiency: correlation with cobalamin absorption

J BELAICHE, J ZITTOUN, J MARQUET, J YVART, AND D CATTAN

From the Service d'Hépato-gastroentérologie, Faculté de Médecine de Créteil, Centre Hospitalier, Villeneuve St Georges, Département d'Hématologie, Hôpital Henri Mondor, Creteil, and the Département de Médecine Nucléaire, Hôpital Bicêtre Kremlin Bicêtre, France

SUMMARY Absorption of cobalamin free or bound to chicken serum was assessed in nine patients with pancreatic insufficiency. Simultaneously the *in vitro* effect of duodenal juice collected from six patients and seven controls was tested on labelled cobalamin complexed to chicken serum or to R salivary binder. Malabsorption of free cobalamin was observed in one of nine patients and in four of nine patients when cobalamin was administered bound to chicken serum. The *in vitro* effect of duodenal juice on cobalamin complexed to chicken serum or to R salivary binder was studied: (a) the percentage of free cobalamin released was significantly decreased in pancreatic insufficiency compared with controls whatever the binder used; (b) the degradation of R salivary binder was different in pancreatic insufficiency and in controls. Despite the *in vitro* abnormalities observed in pancreatic insufficiency, these did not correlate with the *in vivo* absorption of cobalamin which was often normal in our patients.

The exact pathogenesis of cobalamin malabsorption in patients with chronic pancreatitis has not been completely clarified, despite intensive investigations in this field. Most authors consider this malabsorption to be directly related to a defect in the external pancreatic secretion which would be responsible for inadequate degradation of R binders to which cobalamin are bound in gastric juice.[1–5] In normal conditions, different proteases essentially trypsin, degrade R binders and liberate free cobalamin which subsequently complexes to intrinsic factor, unaffected by these enzymes.[2] Several studies *in vivo*[3 5] and *in vitro*[2 6] have shown that in pancreatic insufficiency, R proteins failed to be degraded and sequestered a major fraction of cobalamin and this explained the malabsorption. To this effect, differential absorption of cobalamin bound to intrinsic factor and R proteins was studied using a dual label Schilling test.[7] Cobalamin malabsorption is not a constant feature in patients with pancreatic insufficiency.[8–10] Furthermore, correlation between the *in vitro* undegraded R proteins and the *in vivo* cobalamin absorption has not been fully assessed in these patients. Therefore, we have studied simultaneously in the same patients suffering with pancreatic insufficiency the *in vitro* effect of duodenal juice on two cobalamin binding proteins (chicken serum and saliva) and the absorption of free cobalamin and cobalamin bound to chicken serum.

Address for correspondence: Dr J Belaiche, Hôpital de Villeneuve St Georges, F94190, Villeneuve St Georges, France.

Received for publication 2 May 1986

Methods

PATIENTS

Nine patients with pancreatic insufficiency (chronic pancreatitis, alcohol induced) were investigated. Diagnosis of chronic pancreatitis was established either by the presence of pancreatic calcifications (eight patients) or by an abnormal pancreatogram (one patient). Steatorrhea was the criterium for pancreatic insufficiency. All the subjects investigated in the present study gave their informed consent

COBALAMIN ABSORPTION

The absorption of 0·5 μCi free cobalamin (crystalline (^{57}Co) cyanocobalamin: specific activity 1 μCi/μg) was measured by the standard urinary excretion test.[11] The absorption of protein bound cobalamin was evaluated using cobalamin bound to chicken

serum as previously reported.[12 13] Briefly, the oral dose used in the present study was prepared by mixing 0·5 μCi corresponding to 0·5 μg of (^{57}Co) cyanocobalamin (same specific activity as above) with 2 ml pathogen free chicken serum whose unsaturated B_{12} binding capacity (UB12BC) was 350 ng/ml. A 48 h urine sample was collected and its radioactivity determined and expressed as the percentage of the oral dose.

IN VITRO EFFECT OF DUODENAL JUICE ON COBALAMIN BOUND TO CHICKEN SERUM AND TO SALIVA

Duodenal juice was obtained by direct cannulation of the duodenum under endoscopic visualisation after an intravenous bolus of one Ivy dog unit/kg of pancreozymin (pancreozymin Boots, the Boots company) and of one clinical unit/Kg of secretin (secretin GIH). Fractionated samples were collected in tubes with and without aprotinin (2×10^4 U/ml), a potent inhibitor of proteases, and immediately stored at −30°C. Duodenal juice was obtained in six of the nine patients with pancreatic insufficiency and in seven normal subjects.

Duodenal juice was first saturated with an excess of cold cyanocobalamin then dialysed at 4°C for 24 h against 0·9% saline. 0·4 ml saturated duodenal juice was incubated for four hours at 37°C either with 0·5 ml of 1/40 diluted chicken serum labelled with 4 ng (^{57}Co) cyanocobalamin (Amersham, specific activity: 225 μCi/μg) or with 0·1 ml saliva labelled with 2 ng (^{57}Co) cyanocobalamin of the same specific activity. The UB12BC of chicken serum and saliva used were respectively 350 and 25 ng/ml: in the conditions used, the amount of (^{57}Co) cyanocobalamin bound to the two binders was of the same order of magnitude.

The effect of trypsin (Sigma. type IX porcine pancreas, 15,550 U BAEE/mg) on cobalamin bound to chicken serum and saliva was tested in the same conditions. Five hundred micrograms trypsin in 0·4 ml buffer consisting of Tris-HCl 30 mM, NaCl 0·15 M adjusted to pH 7·5 was incubated for four hours at 37°C with chicken serum and saliva labelled in the same proportions as above.

Digest of the cobalamin binder complex was fractionated by gel filtration on an Ultrogel AcA34 (IBF) 2·6 × 70 cm column. Buffer used was Tris 0·06 M, NaCl M, adjusted to pH 8 with HCl N.

The column was first calibrated with blue dextran to determine the void volume and with albumin, γ-globulins and lysozyme. The molecular weight of chicken serum was assessed by gel chromatography in the same conditions as above and compared with that of saliva, bile, and gastric juice. Immunological reactivity of this binder was tested against an antihuman serum R binder antibody, after incubation with this antiserum and chromatography on Ultrogel.

ANALYSIS OF GASTRIC SECRETIONS

This was carried out in patients with pancreatic disease in basal conditions and after pentagastrin stimulation (6 μg/kg). Hydrogen ion concentration in gastric juice was measured by titration to pH=7·0 with 0·1 N NaOH. Basal acid output and stimulated acid output were calculated. The concentration of intrinsic factor in gastric juice was measured by the method of Gottlieb *et al.*[14] Stimulated intrinsic factor output was calculated. Statistical analysis was performed by the Wilcoxon Mann-Whitney test.

Results

A profile of the nine patients with pancreatic insufficiency is given in Table 1. Steatorrhea ranged

Table 1 *Characterisation and cobalamin absorption of patients with pancreatic insufficiency*

Patient	*Age (yr)*	*Sex*	*Pancreatic calcifications*	*Steatorrhea (g/24h) (n<6)*	*Urinary excretion (%)†*	
					Free cbl (n>10)	*Protein bound cbl (n>0·71)*
1	57	M	*	25	20	0
2	43	M	*	40	19	0
3	41	M	*	90	5·5	0·1
4	38	M	*	22	18	0
5	70	M	*	50	18	17
6	48	M	*	60	34	7·1
7	43	M	No	18	40	8·6
8	43	M	*	34	36	3·8
9	48	M	*	12	22	4·2

Cbl=cobalamin
*Present
†Urinary excretion is expressed as a percentage of the (^{57}Co) cyanocobalamin oral dose

from 12–90 g 24 h with a median of 34 (n<6). Gastric acid secretion was normal in all patients. Stimulated intrinsic factor output was ranged from 1900–21 000 UI/h with a median of 6700 (n>2000).

Cobalamin malabsorption was observed in one patient when the test was done with free cobalamin and in four patients with cobalamin bound to chicken serum (Table 1). The patient with the most important steatorrhea was the only one that had a combined abnormal absorption of both forms.

In the *in vitro* effect of duodenal juice on cobalamin bound to chicken serum and saliva preliminary studies on the characterisation of the cobalamin binder from chicken serum showed that this binder had an apparent molecular weight of 120 000. This molecular weight was similar to that of salivary, biliary, gastric juice R binders (Figure). In addition, the incubation of (^{57}Co) cyanocobalamin bound to chicken serum with an anti-R binder antiserum gave on gel filtration a peak with a larger molecular weight eluted with the void volume, corresponding to an immune complex.

Incubation of chicken serum binder cobalamin complex with duodenal juice from controls induced a release of free cobalamin (median: 1·73% of total radioactivity with a range from 1·5–2·2). This release was significantly lower (p<0·01) when the complex was incubated with duodenal juice originating from patients with pancreatic insufficiency (median: 0·70 ranging from 0·55–0·90) (Table 2). The dissociation of the complex was also significantly decreased when duodenal juice from controls was collected on aprotinin (p<0·01). There was no apparent degradation of the chicken serum binder after incubation with duodenal juice; gel filtration on AcA34 gave identical molecular weight fragments; gel filtration on AcA54 which gives a finer resolution for smaller molecular weight species did not identify any cobalamin binding cleavage products. Incubation of chicken serum with trypsin alone gave results similar to those obtained with normal duodenal juice: R binder and release of free cobalamin in the same proportions.

Incubation of salivary R binder-cobalamin complex with duodenal juice from controls produced a

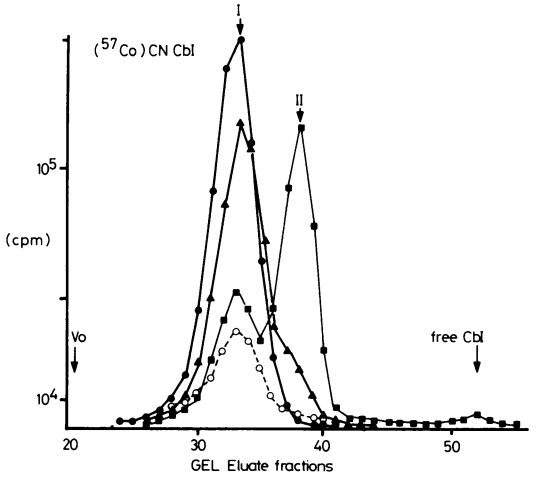

Fig. 1 *The elution pattern of cobalamin binding proteins from chicken serum (▼—▼), bile (○—○), saliva (●—●) and gastric juice (■—■). Peak I: Molecular weight 120 000. Peak II: Molecular weight 55 000 corresponding to intrinsic factor of gastric juice.*

Table 2 *Cobalamin (cbl) released from chicken serum by duodenal juice from normal subjects or from patients with pancreatic insufficiency. Results are expressed in % of total (^{57}Co) cyanocobalamin bound to chicken serum before incubation.*

Subject (n)	*Cbl released*
Normal	
A	2·2
B	1·8
C	1·73
D	1·8
E	1·79
F	1·5
G	1·6
Pancreatic insufficiency	
2	0·6
3	0·55
4	0·7
5	0·73
6	0·9
8	0·75

Table 3 *Release of degraded fragments with various molecular weights (MW) and of free cobalamin (cbl) from cbl-R saliva after incubation with duodenal juice. Results are expressed in % of the total radioactivity.*

Subject (n)	*60 000 MW*	*Degraded salivary R-binder 25 000 to 50 000 MW*	*Cbl released*
Normal			
A	58·5		41·5
B	71		29
C	52		48
F	43		57
G	38		62
Pancreatic insufficiency			
2	—	78	22
3	89	—	11
4	95·5	—	4·5
5	77	—	23
6	58·5	36	5·5
8	—	81	19

degraded fragment of 60 000 molecular weight and cobalamin; no intact R binder was identified (Table 3). Duodenal juice from patients with pancreatic insufficiency incubated with salivary R binder cobalamin showed heterogeneous patterns of degradation. R binder was also degraded either in fragments of 60 000 molecular weight in some patients or in even smaller fragments ranging from 25 000 to 50 000 in other patients. Cobalamin was also released in all patients but in lower proportions than in controls ($p<0{\cdot}05$). Samples of duodenal juice from controls collected on aprotinin induced a poor release of free cobalamin significantly lower than samples without aprotinin ($p<0{\cdot}01$). The major part of the radioactivity was shared between the R binder and the fragment of 60 000 molecular weight when the collection of samples was made on aprotinin.

The action of trypsin on cobalamin bound to saliva was stronger than that of duodenal juice: after four hours of incubation, only cobalamin was identified; whereas on shorter times of incubation the degraded fragment of 60 000 molecular weight was observed concommitant of a release of cobalamin.

Discussion

A malabsorption of free cobalamin was observed in one of the nine patients studied. This frequency is lower than that observed in other series, the incidence of malabsorption rating an average 46%.[8–10] The percentage of malabsorption increased when we assessed the absorption of cobalamin bound to chicken serum (four of nine patients). Another comparative study using cobalamin bound to the R salivary protein failed to show a malabsorption in 11 patients with chronic pancreatitis.[15] Cobalamin malabsorption possibly related to pernicious anaemia or gastric achlorhydria[13] has been excluded because all our patients had normal intrinsic factor output and normal gastric acid secretion.

Our *in vitro* studies showed that duodenal juice from normal subjects induced modifications of cobalamin complexed to chicken serum or to saliva as did those from patients with pancreatic insufficiency. In fact, the cobalamin binding protein present in chicken serum may be assimilated to a R binder as evidenced by the similarity of some of its chemical and immunological properties: reactivity with an antihuman R binder antiserum, with the same molecular weight as that of saliva, gastric juice, and bile R binder.

Our results indicate, however, that the pattern of cobalamin-R binder alterations caused by duodenal juice seem to depend on the source of R binder. Duodenal juice as well as pure trypsin released cobalamin and intact R binder when chicken serum was used as substrate, so that it seems that only the cobalamin binding site is affected. It cannot be excluded, however, that proteolytic fragments would be also produced, although undetectable in our technical conditions, if they cannot bind (^{57}Co) cyanocobalamin. In any case, the percentage of cobalamin released from the chicken binder was significantly decreased in pancreatic insufficiency, compared with controls and there was no overlap between the two groups. The lowest percentage of cobalamin released from chicken serum was observed in patient 3 who presented the greatest degree of steatorrhea. In addition, a malabsorption of both forms of cobalamin was present in this patient. A decreased release of free cobalamin was also observed when normal duodenal juice was collected on aprotinin. The values were close to those observed in pancreatic insufficiency. The presence of intact chicken serum binder shows that this binder is quite resistant to the action of trypsin. This has also been observed with other human R binders.[16 17] Duodenal juice also affected salivary R binders cobalamin complexes but in a different way. Our data show that the saliva R binder was split into smaller fragments. Similar observations have been reported by other investigators using proteolytic enzymes[2] or pure human pancreatic juice.[16] In the controls of our study, duodenal juice degraded R binder into one fragment of 60 000 and free cobalamin, approximatively in the same proportions. In pancreatic insufficiency, the degradation was more heterogeneous according to the patients, but in all cases, the proportion of free cobalamin released was significantly reduced. Intact R binder was split into fragments of 60 000 molecular weight as in controls but also into smaller fragments. Patient 5 in whom the percentage of degraded R salivary was close to that of controls had a normal cobalamin absorption.

Whatever the binder used or whatever the alterations of the cobalamin binder complex induced by duodenal juice, there was a noticeable difference between controls and patients. The percentage of cobalamin released from chicken serum and R saliva and the proportion of degraded fragments from R salivary binder were different in patients with pancreatic insufficiency and in controls. The discrepancy observed between *in vitro* and *in vivo* tests, however, frankly perturbed in one and almost always normal in the other suggests that proteolysis by duodenal juice only partly explains the mechanism involved in cobalamin absorption and that other factors are at work notably in pancreatic insufficiency.

We wish to thank Dr Bruno Schremmer for helpful discussion and for reading the manuscript.

References

1 Okuda K, Kitazaki T, Takamatsu M. Inactivation of vitamin B12 by a binder in rat intestine and the role of intrinsic factor. *Digestion* 1971; **4:** 35–8.

2 Allen RH, Seetharam B, Podell E, Alpers DH. Effect of proteolytic enzymes on the binding of cobalamin to R protein and intrinsic factor. In vitro evidence that a failure to partially degrade R protein is responsible for cobalamin malabsorption in pancreatic insufficiency. *J Clin Invest* 1978; **61:** 47–54.

3 Allen RH, Seetharam B, Allen NC, Podell ER, Alpers DH. Correction of cobalamin malabsorption in pancreatic insufficiency with a cobalamin analogue that binds with high affinity to R protein but not to intrinsic factor. In vivo evidence that a failure to partially degrade R protein is responsible for cobalamin malabsorption in pancreatic insufficiency. *J Clin Invest* 1978; **61:** 1628–34.

4 Andersen KJ, Lippe G Von Der. The effect of proteolytic enzymes on the vitamin B12-binding proteins of human gastric juice and saliva. *Scand J Gastroenterol* 1979; **14:** 833–8.

5 Marcoullis G, Parmentier Y, Nicolas JP, Jimenez M, Gerard P. Cobalamin malabsorption due to non degradation of R proteins in the human intestine inhibited cobalamin absorption in exocrine pancreatic dysfunction. *J Clin Invest* 1980; **66:** 430–40.

6 Guéant JL, Parmentier Y, Djalali M, Bois F, Nicolas JP. Sequestration of crystalline and endogenous cobalamin by R binder down to the distal ileum in exocrine pancreatic dysfunction. *Clin Chim Acta* 1983; **134:** 95–106.

7 Brugge WR, Goff JS, Allen NC. Development of a dual label schilling test for pancreatic exocrine function based on the differential absorption of cobalamin bound to intrinsic factor and R protein. *Gastroenterology* 1980; **78:** 937–49.

8 Toskes PP, Hansell J, Cerda J, Deren JJ. Vitamin B12 malabsorption in chronic pancreatic insufficiency: studies suggesting the presence of a pancreatic "instrinsic factor". *N Engl J Med* 1971; **284:** 627–32.

9 Matuchansky C, Rambaud JC, Modigliani R, Bernier JJ. Vitamin B12 malabsorption in chronic pancreatitis. *Gastroenterology* 1974; **67:** 406–7.

10 Lippe G Von Der, Andersen KJ, Schjonsby H. Intestinal absorption of vitamin B12 in patients with chronic pancreatic insufficiency and the effect of human duodenal juice on the intestinal uptake of vitamin B12. *Scand J Gastroenterol* 1976; **11:** 689–95.

11 Schilling RF. Intrinsic factor studies. II. The effect of gastric juice on the urinary excretion of radioactivity after the oral administration of radioactive vitamin B12. *J Lab Clin Med* 1953; **42:** 860–6.

12 King CE, Leibach J, Toskes PP. Clinically significant vitamin B12 deficiency secondary to malabsorption of protein-bound vitamin B12. *Dig Dis Sci* 1979; **24:** 397–02.

13 Cattan D, Belaiche J, Zittoun J, Yvart J, Chagnon JP, Nurit Y. Rôle de la carence en facteur intrinsèque dans la malabsorption de la vitamine B12 liée aux proteines, dans les achlorhydries. *Gastroenterol Clin Biol* 1982: **6:** 570–5.

14 Gottlieb E, Lau KS, Wasserman LR, Herbert V. Rapid charcoral assay for intrinsic factor (IF) gastric juice antibody to IF and serum unsaturated B12 binding capacity. *Blood* 1965; **97:** 875–84.

15 Hofstad B, Kittang E, Schjonsby H. The effect of R-protein on the absorption vitamin B12 in chronic pancreatitis. [Abstract] *Scand J Gastroenterol* 1983; **18:** suppl. 86: 26.

16 Carmel R, Abramson SB, Renner IG. Characterization of pure human pancreatic juice: cobalamin content, cobalamin-binding proteins and activity against human R binders of various secretions. *Clin Sci* 1983; **64:** 193–05.

17 Kanazawa S, Herbert V. Mechanism of enterohepatic circulation of vitamin B12: movement of vitamin B12 from bile R-binder to intrinsic factor due to the action of pancreatic trypsine. *Trans Assoc Am Physicians* 1983; **96:** 336–44.